REMARKS

Claims 1, 3, 14, and 43-50 constitute the pending claims in the present application. The Office Action has acknowledged the amendments and arguments filed on January 16, 2004 and February 11, 2004. Applicants respectfully request reconsideration in view of the following remarks.

Claim rejections under 35 USC §103(a)

Claims 1, 3, 14, 43-49 are rejected under 35 U.S.C. 103 as being unpatentable over Girasole *et al.* in view of Kishimoto *et al.* (U.S. Pat. No. 5,888,510).

The Office Action contends that Girasole discloses administering a neutralizing antibody and a monoclonal antibody (mAb) to IL-11 to inhibit osteoclast formation *in vitro*. As argued before, the Office Action admits that Girasole fails to disclose a method of administering *in vivo* to any mammalian patient such antibodies; and more importantly, Girasole fails to teach an increase in osteoblast-mediated bone formation when an IL-11 antibody is administered to patients having a pathological condition in which bone density is decreased.

The Office Action also suggests that Kishimoto teaches a method for inhibiting synovial cell growth, or for treating chronic rheumatoid arthritis (RA), by administering to a patient polyclonal or monoclonal antibodies to IL-6 or IL-6 receptor, thus disrupting IL-6 / IL-6 receptor binding and IL-6 signaling. Thus, the Office Action concludes that "...if a cytokine causes a disease, and antibody to the cytokine will block the signal transduction by the cytokine, inhibit the cytokine's biological activity and has an alleviating and therapeutic effect on the symptoms of the disease" (emphasis added).

Based on these, the Office Action asserts that it would have been *prima facie* obvious to a skilled artisan, "from the Girasole method to administer IL-11 Ab to patients as taught by Kishimoto *et al.*, to obtain the known functions and advantages thereof as per the teaching of both Girasole and Kishimoto." Applicants respectfully disagree for the following reasons.

Kishimoto presumably establishes that IL-6 signaling *in vivo* is responsible for chronic rheumatoid arthritis (RA). In other words, Kishimoto presumably proves that excessive amount of IL-6 causes RA in a patient. Therefore, there may be a nexus between administering an anti-IL-6

antibody *in vivo* to an RA patient, and the ability of the same anti-IL-6 antibody to block IL-6 signaling *in vitro*.

But as explained before, it is not at all clear, at the time of the instant invention, whether IL-11 signaling *in vivo* is responsible for bone loss, or whether excessive amount of IL-11 causes bone loss in a patient. Therefore, even if there might be a nexus between inhibiting osteoclast function *in vitro* and inhibiting osteoclast function *in vivo*, there is no nexus between inhibiting osteoclast function *in vitro* and inhibiting bone loss *in vivo*. A skilled artisan would not know whether IL-11 indeed causes bone density loss *in vivo* for the following two reasons.

First of all, at the time of the instant invention, it was known that IL-11 stimulates osteoclast function by eliciting IL-11 signaling in osteoclasts (see the Girasole article). So it is possible that IL-11 can stimulate bone resorption. But it was also known, at the time of the instant invention, that osteoblasts contain IL-11 receptor (see last sentence in the abstract of Romas *et al.*, *J. Exp. Med.* **183**: 2581-2591, 1995, **Exhibit A**). Thus, it is also possible that IL-11 can stimulate bone formation. Given the opposite functions of osteoclast and osteoblast *in vivo*, a skilled artisan would not know whether IL-11 administration to a patient *in vivo* would actually cause net bone formation or bone loss. This is completely different from IL-6 signaling, where it is presumably shown that IL-6 act on a single cell type (synovial cells) to cause RA.

Secondly, at the time of the instant invention, it is unknown whether IL-11 signaling would have a significant effect on bone density *in vivo*. A plethora of cytokines / growth factors were known to be able to regulate at least one aspect of bone homeostasis - osteoclastogenesis. Simply because a cytokine (e.g. IL-11) can perform a certain function *in vitro*, does not necessarily mean that the cytokine is the sole or even a significant cytokine that performs that function *in vivo*. Thus, it is completely possible that IL-11 is capable of affect bone density, but only in such a minor role that eliminating this effect *in vivo* would have no discernable changes in overall bone density. This is analogous to the situation where several streams flow into a pond, cutting off any single stream, especially the small ones, may not have any appreciatable effect on water level in the pond at all.

Thirdly, the nexus between IL-6 and IL-11 – both cytokines, as used herein by the Office Action – is too casual and loose to provide a strong enough motivation to combine and sound enough predictability (expectation of success) to a skilled artisan. "Cytokine" is a very broad

functional term (defined as "any of various proteins secreted by cells of the immune system that serve to regulate the immune system" by the *Merriam-Webster Medical Dictionary*) used to refer to a very large family of secreted proteins with extremely broad spectrum of biological functions, but not necessarily any structural similarities. For example, in the instant case the condition related to IL-6 in Kishimoto is RA *in vivo*, while the condition related to IL-11 in Girasole is inhibition of osteoclast function *in vitro*, which has nothing to do with RA. Plus there is no obvious structural similarity between IL-6 and IL-11. Therefore, a skilled artisan would not be motivated to rely on the result from a totally unrelated protein in a totally unrelated function to predict the effect of inhibiting IL-11 *in vivo*, simply because these two unrelated proteins all belong to the loose family called "cytokines." If the name "IL-6" and "IL-11" sound like there is an actual connection, IL-6 is also known in the art as "interferon beta-2," "B-cell differentiation factor," "B-cell stimulatory factor 2 (BSF2)," "hepatocyte stimulatory factor (HSF)," or "hybridoma growth factor (HGF)."

Another problem with cytokine is redundancy – many structurally similar or different cytokines might unexpectedly regulate the same biological function, and neither one of them is "essential" for the regulated function. In other words, taking away any one of the many cytokines regulating the same biological function (such as bone formation or resorption) may or may not have a discernable effect on that biological function. Thus there is no reasonable expectation of success without actually doing the experiment with the particular cytokine.

This problem is also linked to the problem of *in vitro – in vivo* correlation for cytokines. Very often, a cytokine is found to have the ability to affect a biological function (such as regulating osteoclast function) *in vitro*, but turns out to be insignificant *in vivo* due to the fact that other cytokines can carry its "duty" at the absence of that cytokine. An example on point is shown by Nandurkar *et al.* (*Blood* **90**: 2148-2159, 1997, see **Exhibit B** in response to the Office Action mailed on July 16, 2001), who demonstrated, surprisingly, that mice lacking IL-11R showed wild-type level of adult hematopoiesis, although it was known that IL-11 affects hematopoiesis *in vitro*. This indicated that preventing the function of IL-11R *in vivo*, as accomplished by Nandurkar *et al.* by "knocking out" the IL-11R, may not achieve the same effect seen *in vitro*.

Because of these, a skilled artisan would not see Kishimoto as a reference relevant to the problem of treating bone loss *in vivo*, and thus would not be motivated to combine Girasole with Kishimoto. It may be obvious to block the function of a cytokine, if it is known that the cytokine

indeed causes the disease plaguing the patient. But here, until the surprising finding of the instant invention that IL-11 actually has *opposite* effects on osteoclast and osteoblast, and that IL-11 signaling *does* affect bone density in animal model, no one is certain about the *in vivo* effect (and thus usefulness) of IL-11 in treating bone loss patients.

In fact, Applicants note that prior arts in Kishimoto (Wendling *et al.*, "Treatment of severe Rheumatoid Arthritis By anti-IL-6 monoclonal Antibody," *J. of Rheumatology* **20**: 259-262, 1993) established that administering IL-6 antibody to chronic RA patients was at least temporary beneficial in treating RA (see column 2, lines 31-35 of Kishimoto). Meanwhile, Kishimoto at best clarified the mechanism of IL-6 signaling in RA (blocking IL-6 function with IL-6 antibody in synovial cells is beneficial for RA patients). If such a clarification of mechanism of a known method is non-obvious and patentable (see claims 6-11 of Kishimoto), the pending claims of the instant application, based on a novel and unobvious mechanism and use of the IL-11 Ab, ought to be novel and unobvious and thus patentable.

Claim 50 is rejected under 35 U.S.C. 103 as being unpatentable over Girasole *et al.* (1995) in view of Kishimoto (supra) and Queen *et al.* (U.S. Pat. No. 5,530,101).

Girasole and Kishimoto are discussed above. The Office Action alleges that Queen teaches humanization of monoclonal antibodies, thus rendering claim 50 obvious when combined with Girasole and Kishimoto.

As argued above, the claimed invention is not obvious in view of Girasole and Kishimoto, and Queen does not in anyway correct these defects, even assuming a skilled artisan would be motivated to combine Queen with Girasole and Kishimoto to make humanized antibodies to inhibit osteoclast function.

Pursuant to MPEP 2143, "To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the reference themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations."

Therefore, none of the three requirement for establishing a *prima facie* case of obviousness is met. Reconsideration and withdrawal of rejection under 35 U.S.C. 103(a) are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,

Date: April 20, 2004

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The Role of gp130-mediated Signals in Osteoclast Development: Regulation of Interleukin 11 Production by Osteoblasts and Distribution of Its Receptor in Bone Marrow Cultures

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Summary

Interleukin (IL)-11 is a multifunctional cytokine whose role in osteoclast development has not been fully elucidated. We examined IL-11 production by primary osteoblasts and the effects of rat monoclonal anti-mouse glycoprotein 130 (gp130) antibody on osteoclast formation, using a coculture of mouse osteoblasts and bone marrow cells. IL-1, TNFα, PGE_2, parathyroid hormone (PTH) and 1α,25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D_3$) similarly induced production of IL-11 by osteoblasts, but IL-6, IL-4, and TGFβ did not. Primary osteoblasts constitutively expressed mRNAs for both IL-11 receptor (IL-11Rα) and gp130. Osteotropic factors did not modulate IL-11Rα mRNA at 24 h, but steady-state gp130 mRNA expression in osteoblasts was upregulated by $1\alpha,25(OH)_2D_3$, PTH, or IL-1. In cocultures, the formation of multinucleated osteoclast-like cells (OCLs) in response to IL-11, or IL-6 together with its soluble IL-6 receptor was dose-dependently inhibited by rat monoclonal anti-mouse gp130 antibody. Furthermore, adding anti-gp130 antibody abolished OCL formation induced by IL-1, and partially inhibited OCL formation induced by PGE_2, PTH, or $1\alpha,25(OH)_2D_3$. During osteoclast formation in marrow cultures, a sequential relationship existed between the expression of calcitonin receptor mRNA and IL-11Rα mRNA. Osteoblasts as well as OCLs expressed transcripts for IL-11Rα, as indicated by RT-PCR analysis and in situ hybridization. These results suggest a central role of gp130-coupled cytokines, especially IL-11, in osteoclast development. Since osteoblasts and mature osteoclasts expressed IL-11Rα mRNA, both bone-forming and bone-resorbing cells are potential targets of IL-11.

Interleukin (IL)-11 is a functionally pleiotropic cytokine that was isolated from a bone marrow–derived stromal cell line based on its ability to stimulate the proliferation of IL-6–dependent cells (1). An indication that IL-11 might regulate connective tissue responses was given by its expression in relatively restricted cells of the mesenchymal lineage, such as lung fibroblasts, bone marrow stromal cells, placental stromal cells, articular chondrocytes, and synoviocytes (1–4).

The IL-11 receptor is a cell surface receptor that consists of two components: a unique ligand-binding 150-kD glycoprotein chain (IL-11Rα) (5, 6) and a non-ligand binding, signal transducing 130-kD glycoprotein chain (gp130)[1] (7, 8). Both components are necessary for high affinity binding and signal transduction (6, 8). Signaling occurs after ligand-induced dimerization of gp130 that activates down-

A portion of this work was presented at the 17th Annual Meeting of the American Society for Bone and Mineral Research (Baltimore, MD, September 9–13, 1995) and has been published in an abstract form (1995. *J. Bone. Miner. Res.* 10:S142).

[1] *Abbreviations used in this paper:* $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D3; CTR, calcitonin receptor; gp130, glycoprotein 130; IL-11Rα, IL-11 receptor; IL-6Rα, IL-6 receptor; LIF, leukemia inhibitory factor; OCL, osteoclast-like multinucleated cell; OSM, oncostatin M; PTH, parathyroid hormone; sIL-6Rα, soluble IL-6 receptor; TRAP, Tartrate-resistant acid phosphatase.

stream molecules. These include members of the JAK family of non-receptor kinases and a latent transcription factor, acute phase response factor or signal transducer and activator of transcription factor 3 (APRF/STAT 3) (9, 10). The gp130 signal transducer mediates biological effects exerted not only by IL-11, but also IL-6, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (11, 12).

Several lines of evidence suggest that IL-11 is an important osteotropic factor. IL-11 receptor transcripts are present in chondroblastic and osteoblastic progenitor cells during mouse embryogenesis (13). IL-11 itself is produced by human osteosarcoma SaOs-2 cells (14), and Girasole et al. (15) showed that IL-11 dose-dependently stimulated osteoclast-like multinucleated cell (OCL) formation in cocultures of mouse osteoblasts and bone marrow cells. They reported that monoclonal anti-IL-11 antibody inhibited osteoclast formation induced by several osteotropic factors. We also reported that IL-11, IL-6 together with its soluble IL-6 receptor (sIL-6Rα), OSM and LIF similarly triggered OCL formation in cocultures (16, 17).

These observations focus interest on the modulation of IL-11 and its receptor during OCL development. In the present study, we measured IL-11 production by primary mouse calvarial cell cultures using a novel bioassay based on the expression of functional IL-11 receptors in Ba/F3 cells (6). We examined the effects of IL-1, TNFα, PGE_2, parathyroid hormone (PTH) and 1α,25-dihydroxyvitamin D3 [$1\alpha,25(OH)_2D_3$], all of which induce OCL formation in mouse cocultures. The role of gp130-mediated signals in OCL formation was determined using a rat monoclonal anti-mouse gp130 antibody. The expression of gp130 and IL-11Rα mRNA was studied by Northern analysis and reverse transcription-PCR, respectively. The distribution of IL-11Rα transcripts was investigated by in situ hybridization.

We found that osteotropic factors induced IL-11 production by osteoblasts and in addition, modulated steady-state levels of osteoblast gp130 mRNA. Interleukin-11 receptor transcripts were detected in osteoblasts and osteoclast-like cells. In the present study we demonstrate that gp130-mediated signals are critical cofactors for osteoclastogenesis induced by a variety of bone resorbing factors.

Materials and Methods

Animals and Cytokines. Newborn and 6–9-wk-old male C57/B16J mice were purchased from Monash University Animal Services Center (Clayton, Australia). Recombinant human IL-11 (specific activity 10^8 U/mg protein) was obtained from Dr. T. Willson (Walter and Eliza Hall Institute, Melbourne, Australia). Neutralizing monoclonal murine anti-human IL-11 antibody was generously provided by Genetics Institute (Cambridge, MA). Recombinant mIL-11, mIL-6, hOSM, mIL-3, mIL-4, hLIF, mIL-1α, and mTNFα were purchased from Peprotech (Rocky Hill, NJ). Mouse sIL-6R was prepared from CHO cells as described (16). Recombinant hTGFβ1 was the gift of Genentech Inc. (San Francisco, CA). $1\alpha,25(OH)_2D_3$ was obtained from Wako Pure Chemical Co. (Osaka, Japan). Bacterial collagenase was obtained from Worthington Biochemical Co. (Freefold, Australia). Other chemicals and reagents were of analytical grade.

Cell Culture and Determination of Osteoclast Characteristics. To isolate osteoblastic cells, 6–10 calvaria obtained from 1-d-old mice were cut into small pieces in 10-cm culture dishes with 5 ml of α-MEM (GIBCO BRL, Gaithersburg, MD) containing 30% FBS (Cytosystems, Castle Hill, NSW, Australia). Type-I collagen gel solution (70%, 5 ml) (Cellmatrix Type I-A; Nitta Gelatin Co., Osaka, Japan) was added in the culture dish according to the manufacturers' instructions. After culture for 4–6 d, osteoblastic cells grown from the calvaria were collected by treating with PBS containing 0.3% collagenase for 20 min at 37°C (17). These freshly isolated osteoblastic cells were cocultured with bone marrow cells as described (16, 17). In short, primary osteoblastic cells (10^4 per well) and nucleated marrow cells (2×10^5 per well) were cocultured in 48-well plates (Corning Glass Ins., Corning, NY) with 0.3 ml of α-MEM containing 10% FBS in the presence of test chemicals. Cultures were incubated in quadruplicate and cells were replenished on day 4 with fresh medium. OCL formation was evaluated after culturing for 6 d. For tartrate-resistant acid phosphatase (TRAP) staining, adherent cells were fixed with 4% formaldehyde in PBS for 3 min. After treatment with ethanol-acetone (50/50, vol/vol) for 1 min, the well surface was air dried and incubated for 10 min at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% napthol AS-MX phosphate (Sigma Chem. Co., St. Louis, MO) as a substrate and 0.03% red violet LB salt (Sigma) as a stain for the reaction product in the presence of 50 mM sodium tartrate. TRAP positive cells appeared as dark red cells. The expression of calcitonin receptors (CTR) was assessed by autoradiography using [^{125}I]-salmon calcitonin as described (16–20).

Mouse bone marrow cell cultures were prepared as described (18, 20). The marrow cells were plated at 1×10^6 cells/ml and incubated in α-MEM containing 12.5% FBS in 9-cm petri dishes (Nunc, Inc. Naperville, IL). OCL formation was induced by $1\alpha,25(OH)_2D_3$ (10 nM), added on the first day and every third day thereafter, when half the medium was replaced with fresh medium. In parallel cultures, the cells were cultured on top of 13-mm Thermanox coverslips (Nunc) for enumeration of OCLs by TRAP-staining and [^{125}I]-calcitonin binding.

For in situ hybridization, after coculturing on collagen gels for 7 d (19), cells were treated with collagenase (0.2%), and subcultured on top of 13-mm diameter Thermanox coverslips (Nunc) in 24-well plates, before processing.

A stock of the mouse bone marrow-derived stromal cell line ST2 (21, 22), was obtained from RIKEN cell bank (Tsukuba, Japan). ST2 cells in the 12th passage were grown to sub-confluence in 75-cm^2 tissue culture flasks (Nunc), in phenol red-free α-MEM containing 10% FBS. The medium was changed to 2% FBS overnight, and cells were treated with osteotropic factors before extraction of total RNA at the appropriate times.

Biological Assay for IL-11. IL-11 activity was measured using the Ba/F3 cell microproliferation assay (6, 23). Survival and proliferation of Ba/F3 cells are supported by IL-3, and also by IL-11 after transfection with cDNAs for mIL-11Rα and gp130. Transfected cells also respond to high concentrations of the complex of mIL-6 and sIL6Rα (3 μg/ml IL-6 and 500 ng/ml sIL-6Rα, respectively), but do not proliferate in response to OSM, IL-6, or LIF (6). The cells did not show effects with other cytokines or reagents used in the present study. Ba/F3 clones were grown in DME containing 10% (vol/vol) FBS and 10% (vol/vol) WEHI-3B D-conditioned medium as a source of IL-3 (24). Cells were

maintained by twice weekly passage and used in exponential growth phase. Cell proliferation was measured in Lux-60 microwell HL-A plates (Nunc). For use, Ba/F3 cells were washed three times in DME containing 20% (vol/vol) FBS and resuspended at a concentration of 20,000 cells/ml in the same medium. 10-μl aliquots of cell suspension were then placed in the culture wells with 5 μl of serial dilutions of recombinant IL-3, IL-11, or culture supernatants. After 2 d of incubation at 37°C in a fully humidified incubator containing 10% CO_2 in air, viable cells were counted using an inverted microscope. IL-11 concentrations in the supernatants were determined from a standard curve set up with known amounts of recombinant IL-11. The detection limit of this assay was typically 50–100 pg/ml (Fig. 1).

ELISA for sIL6Rα. Concentrations of sIL-6Rα were determined by a sandwich ELISA using rat monoclonal anti-mIL-6Rα antibody and rabbit polyclonal anti-mIL-6Rα antibody, as described (25). The detection limit of this assay was 1 ng/ml.

Northern Analysis. Total cellular RNA was extracted using the guanidine thiocyanate-phenol chloroform method (26), fractionated by electrophoresis on 1.5% agarose-formaldehyde gels (20 μg/lane), and transferred to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL). A 2.7-kb mouse gp130 cDNA (27) was random prime labeled with [^{32}P]dCTP to a specific activity of 1 × 10^9 U/min/mg DNA (Boehringer Mannheim, Mannheim GmbH, Germany). Hybridization and washing were carried out as previously described (28). Specifically bound cDNA was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnydale, CA). Relative mRNA levels were normalized for loading variability by comparison with 18S ribosomal RNA levels in the same membranes, probed with a ^{32}P-labeled 18S ribosomal RNA oligonucleotide.

PCR Amplification of Reverse-transcribed mRNA. Expression of IL-11Rα, IL-6Rα, and calcitonin receptor (CTR) mRNA reverse-transcribed from primary osteoblasts or bone marrow cell cultures were determined by PCR and Southern blot analysis. First strand cDNA was synthesized from 2.5 μg of total RNA by incubation for 1 h at 42°C with 12 U of avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) after random hexanucleotide priming. The reaction mixture (25 μl) was made up to 100 μl with sterile distilled water, and 10 μl (1/10) were submitted for PCR to amplify the sequences of the mouse IL-11Rα, IL-6Rα, CTR, and GAPDH mRNAs specified below. The reaction mixture contained 50 pmol of each primer, 0.25 mM dATP, dGTP, dCTP, and dTTP (Pharmacia, Uppsala, Sweden), 2 μl 10× reaction buffer, 1 U of Taq DNA polymerase (Boehringer Mannheim) and sterile distilled water, and was overlaid with 50 μl of paraffin oil. PCR for CTR, IL-11Rα, and IL-6Rα was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus Instrs., Norwalk, CT) as described (17). To permit semiquantitation of the PCR products, preliminary experiments were performed to ensure that the number of PCR cycles employed for each product in a given experiment (described in the legends to Figs. 5 and 9) was within the exponential phase of the amplification curve. PCR products were resolved on a 2% wt/vol agarose gel and the specificity of the reaction was confirmed by Southern transfer onto nylon membranes (Hybond-N; Amersham) and hybridization with ^{32}P-labeled internal oligonucleotide probes.

Control PCR reactions were carried out on non-reverse transcribed RNA; in none of these samples were PCR products detected.

Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 381A (Foster City, CA). The oligonucleotides for mouse IL-6Rα (17) were: 5′-CCTGTGTGGGGTTCCAGAGGAT-3′ (3′ primer complementary to nucleotides 980–1001) and 5′-CTGCCAGTATTCTCAGCAGCTG-3′ (5′ primer complementary to nucleotides 488–519). The products were verified by Southern hybridization with the internal oligonucleotide, 5′-CACAACGAAGCGTTTCACAGCTT-3′. The oligonucleotides for mouse IL-11Rα (6, 17) were: 5′-GGAGGCCTCCAGAGGGT-3′ (3′ primer complementary to nucleotides 661–667) and 5′-GGGTCCTCCAGGGGTCCAGTATGG-3′ (5′ primer complementary to nucleotides 133–156). The products were verified by Southern hybridization with the internal oligonucleotide, 5′-CTCCTGTACTTGGAGTCCAGG-3′. The oligonucleotides for mouse CTR were: 5′-ACAAACTGGA(T/C)(T/G)CCCAGCAGGGGCAC-3′ (3′ primer complementary to nucleotides 1663–1689) and 5′-AAGAACATGTT(C/T)CT(C/G/T)ACTTA (5′ primer complementary to nucleotides 1058–1079), as we previously reported (20). The CTR PCR products were verified with the internal sense strand oligonucleotide, 5′-ACCAAGATGAGGCAAACC-3′, by Southern hybridization.

To ensure equal starting quantities of cDNA for the experiments, and to allow semiquantitation of the PCR products representing mIL11-Rα or mIL-6Rα, the reverse-transcribed RNA samples were also amplified using oligonucleotide primers specific for GAPDH (29). A fragment of ~420 bp was amplified using 5′-specific oligonucleotide, (5′-CATGGAGAAGGCTGGGGCTC-3′, representing nucleotides 306–325 of rat GAPDH) and 3′-specific oligonucleotide, (5′-AACGGATACATTGGGGGTAG-3′, representing nucleotides 701–720). Products were verified with [^{32}P]-labeled internal oligonucleotide (5′-GCTGTGGGCAAGGTCATCCC-3′, representing nucleotides 640–659). The signals were quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Preparation of Anti-Mouse gp-130 Antibody. Wistar rats were immunized with 100 μg of soluble mouse gp130 in Freund's complete adjuvant, followed by seven 50-μg boosts of soluble mouse gp130 in Freund's incomplete adjuvant once a week. 3 d after the last boost, the rats were sacrificed and spleen cells were fused with P3U1 mouse myeloma cells with polyethylene glycol 1500 (Boehringer Mannheim). Hybridomas were established by conventional hypoxanthine/aminopterin/thymidine selection methods, and hybridomas producing anti-mouse gp130 were selected by flow cytometry using BAF-m130 cells, which were established by transfection of BAF-B03 cells with mouse gp130 cDNA (27).

Briefly, culture supernatants of the hybridomas (100 μl) were added to BAF-m130 cells (5 × 10^5 cells) with 100 μl of Ca^{2+}, Mg^{2+}-free phosphate-buffered saline containing 2% heat-inactivated FBS and 0.1% sodium azide. The cells were incubated for 20 min on ice, and then incubated for 20 min with FITC-conjugated mouse anti-rat IgG (2 μg/ml) on ice. Stained cells were analyzed by flow cytometry using FACScan® (Becton Dickinson Immunocytometry Sys., Mountain View, CA). Hybridomas recognizing soluble mouse gp130 were cloned twice by limiting dilution. Established hybridomas were expanded as ascite tumors in BALB/C nude mice, and the antibody was purified from the fluid with protein G-agarose column (Oncogene Science, Inc., Manhasset, NY).

Synthesis of Riboprobe. A riboprobe for mouse IL-11Rα was synthesized and labeled with digoxigenin (DIG) by using a RNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. A 1350-bp PCR fragment in the BsoXI site of the mammalian expression vector pEF-BOS (6) was subcloned into the XbaI site of pBluescript (Stratagene, Inc., La Jolla, CA). The antisense and sense probes were then obtained after linearization of the plasmid with SmaI for T3 and NotI for T7 RNA poly-

merase transcription, respectively. The specificity of the cRNA probe for its target sequence was verified by Northern analysis of total RNA derived from transfected Ba/F3 cells.

In Situ Hybridization. In situ hybridization was performed as described with minor modifications (30). Cocultures established on top of 13-mm coverslips in 24-well plates were rinsed in ice-cold PBS, and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min. After washing in PBS, they were pretreated with 0.2 M HCl for 20 min, washed with sterile distilled water for 10 min, and digested with 5 μg/ml proteinase K (37°C; 30 min) in PBS. After treatment with 2 mg/ml of glycine in PBS to inactivate proteinase K, they were refixed with 4% PFA-PBS for 15 min. Hybridization was carried out with 2–4 ng/μl of antisense cRNA in a damp chamber in a solution containing 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim), 0.02% sodium dodecyl sulfate, and 0.1% *N*-lauroylsarcosyl, for 18 h at 42°C. After hybridization, coverslips were washed sequentially with 2× SSC for 30 min, treated with 20 μg/ml ribonuclease (RNase, DNase free; Boehringer Mannheim) in 2× SSC for 30 min to remove excess cRNA, then washed with 1× SSC for 30 min, and finally with 0.1× SSC for 30 min. Detection of hybridized probe was with the anti-DIG-alkaline phosphatase conjugate, according to the manufacturer's instructions. The coverslips were rehydrated in ethanol, and counterstained with nuclear fast red before mounting.

Cytospin preparations of transfected Ba/F3 cells served as positive controls. Hybridization specificity was verified by elimination of signals after pretreatment with 100 μg/ml of RNase for 1 h before hybridization with antisense riboprobe.

Statistical Analysis. Statistical significance was determined by Student's *t* test.

Results

Regulation of IL-11 Production by Primary Murine Osteoblasts. We first analyzed IL-11 production by primary osteoblasts derived from mouse calvaria. Subconfluent osteoblasts were incubated in α-MEM containing 10% FBS in the presence or absence of osteotropic factors, and IL-11 activity in the culture supernatants was quantitated using the Ba/F3 bioassay. Fig. 1 shows the effect of graded concentrations of rhIL-11 on the viability of transfected Ba/F3 cells expressing the mIL-11Rα chain and gp130 (*left*), and the neutralization of this response by monoclonal murine anti-human IL-11 antibody (αIL-11 mAb, *right*). Parental Ba/F3 cells did not respond to rhIL-11, whereas both parental and transfected cells proliferated in response to rmIL-3 (data not shown). The specificity of this microassay depended on the parallel response of transfected and parental cells.

Using this assay, it was clear that unstimulated osteoblasts produced levels of IL-11 that were below the limits of detection in our assays. In contrast, cells that were stimulated with mIL-1α, mTNFα, PGE_2, PTH, and $1\alpha,25(OH)_2D_3$ produced readily detectable levels of IL-11. As seen in Fig. 2, the time-dependent effects of IL-1, TNFα, or PGE_2 were appreciated only after incubation for 6 h, and were near-maximal after 12-h incubation. The levels of IL-11 present after PTH or $1\alpha,25(OH)_2D_3$ stimulation were not maximal before 24 h.

Fig. 3 demonstrates the dose-dependent nature of these

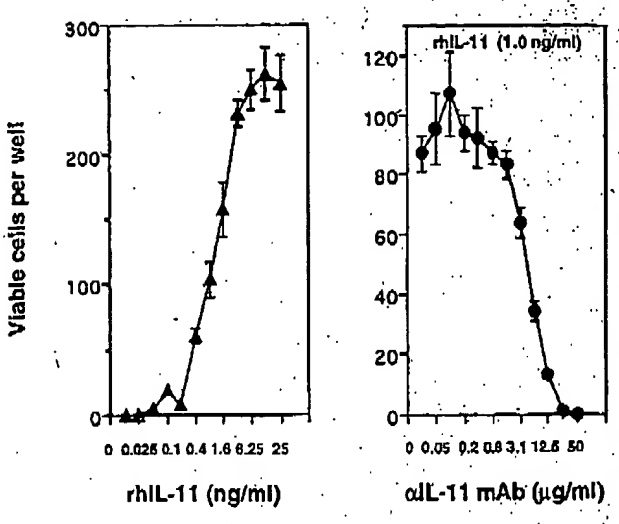

Figure 1. Left panel shows a microassay for Ba/F3 cell proliferation. Transfected Ba/F3 cells expressing the mIL-11Rα chain and gp130 were incubated at 200 cells per well in a volume of 15 μl with the indicated amounts of rhIL-11. After incubation for 48 h the numbers of viable cells were counted. In parallel incubations, parental Ba/F3 cells were non-viable (not shown). Right panel shows the effect of graded concentrations of monoclonal murine anti-human IL-11 antibody (αIL-11 mAb) on the response of transfected Ba/F3 cells to rhIL-11 (1.0 ng/ml).

responses. The effects of IL-1 or TNF were evident with concentrations as low as 0.1 ng/ml (the lowest concentration tested). Doses as low as 10 pM $1\alpha,25(OH)_2D_3$ and 25 ng/ml of hPTH (1–34) induced IL-11. Importantly, the ability to induce IL-11 release from osteoblasts was not a general property of all osteotropic cytokines, since mIL-6 (20 ng/ml), hOSM (20 ng/ml), hTGFβ (1 ng/ml), or mIL-4 (10 ng/ml) did not elicit IL-11 release (Fig. 3). The effects of IL-1 and TNFα on IL-11 production were abolished by incubating with the cyclooxygenase inhibitor indomethacin (10^{-6} M), but the effects of PGE_2, PTH, or $1\alpha,25(OH)_2D_3$ were not (data not shown). Thus, prostaglandins seemed to be involved in IL-11 production induced by IL-1 and TNF in these cultures. Indeed PGE_2 itself potently induced IL-11 production by osteoblasts (Figs. 2 and 3).

In view of the possibility that sIL-6Rα present in conjunction with IL-6 might also have stimulated the transfected Ba/F3 cells, we assayed the sIL-6Rα concentrations by ELISA. In none of the culture supernatants from these experiments was sIL-6Rα detectable (detection limit 1 ng/ml; data not shown). In contrast, the growth activity in the supernatants was completely neutralized by monoclonal murine anti-human IL-11 antibody (Table 1). Together, this evidence indicated that the activity in the culture supernatants measured by the microassay was due to the presence of IL-11.

Endogenous IL-11 Production in Cocultures of Mouse Bone Marrow Cells and Primary Osteoblasts. In view of the importance of $1\alpha,25(OH)_2D_3$ in mediating osteoclastogenesis in the mouse coculture system (31–33), and the apparent ability of monoclonal anti-human IL-11 antibody to abrogate osteoclast formation (15), we measured the effect of $1\alpha,25(OH)_2D_3$

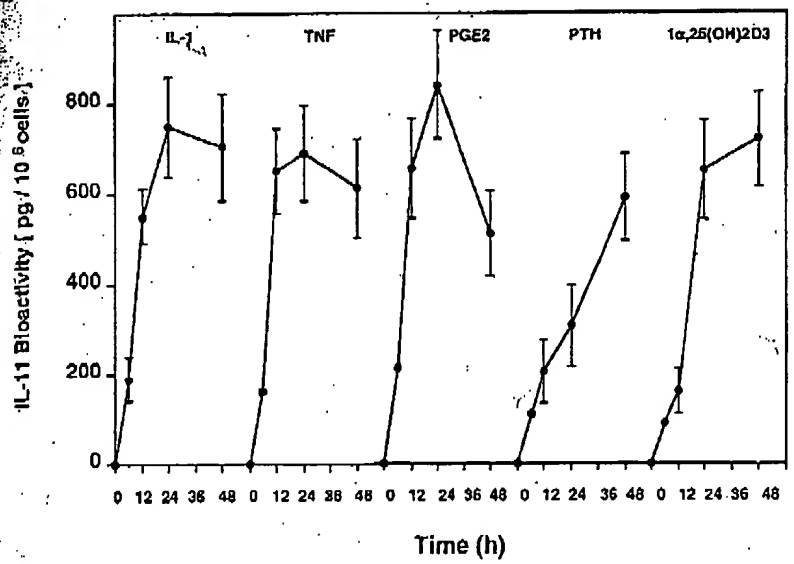

Figure 2. IL-11 production by primary osteoblasts. Subconfluent murine osteoblasts were cultured for up to 48 h in the absence (unstimulated) or presence of mIL-1α (5 ng/ml), mTNFα (10 ng/ml), PGE_2 (10^{-6} M), PTH (200 ng/ml), or $1\alpha,25(OH)_2D_3$ (10 nM). IL-11 released into the supernatants was quantitated at each time point using the Ba/F3 cell bioassay. Each value represents the IL-11 bioactivity (mean ± SE) of triplicate cultures. Unstimulated osteoblasts did not release detectable IL-11 at any time point (data not shown). These results are representatives of replicate experiments.

on endogenous IL-11 production in cocultures. As shown in Fig. 4, treatment with 10 nM $1\alpha,25(OH)_2D_3$ resulted in striking accumulation of IL-11 in the supernatants, significantly different from untreated cocultures, in which IL-11 was not detected.

Expression of Transcripts for IL-11Rα Chain and gp130 by Primary Osteoblasts. Since functional responses to IL-11 are dependent not only on local production of the cytokine but also expression of its specific receptors, we next determined expression of IL-11 receptor mRNAs by primary osteoblasts. As shown in Fig. 5, these cells constitutively expressed transcripts for IL-11Rα and gp130. By the semi-quantitative RT-PCR procedure, no modulation of IL-11Rα mRNA relative to GAPDH mRNA was apparent after 24 h stimulation with $1\alpha,25(OH)_2D_3$, PTH, or IL-1. However, Northern analysis revealed that steady-state gp130 mRNA levels were upregulated. Thus, after 24 h $1\alpha,25(OH)_2D_3$ (10 nM) and PTH (200 ng/ml) elevated gp130 mRNA levels 3.1- and 1.7-fold above control, respectively. Similarly, IL-1 (10 ng/ml) upregulated gp130 mRNA levels 2.4-fold above unstimulated osteoblasts. $1\alpha,25(OH)_2D_3$ also upregulated gp130 mRNA in ST2 cells, a clonal mouse stromal cell line that is capable of supporting osteoclast formation (21, 22). In contrast, other steroid hormones (β-Estradiol or Dexamethasone) did not affect steady-state gp130 mRNA in ST2 cells (Fig. 6).

Effects of Murine Monoclonal Anti-gp130 Antibody on Osteoclast Formation. To establish that gp130-mediated signal transduction is required for osteoclast formation by IL-6 or IL-11, we prepared a rat monoclonal anti-mouse gp130 antibody. The antibody was used to define the role of gp130-mediated signals in osteoclast formation. As shown in Fig. 7, graded concentrations of gp130 antibody added to murine cocultures dose-dependently inhibited osteoclast formation induced by mIL-6 (20 ng/ml) in combination with smIL-6Rα (500 ng/ml), or IL-11 (10 ng/ml). We established that 10 μg/ml of the antibody completely neutralized the osteoclastogenic activity of each cytokine. More importantly,

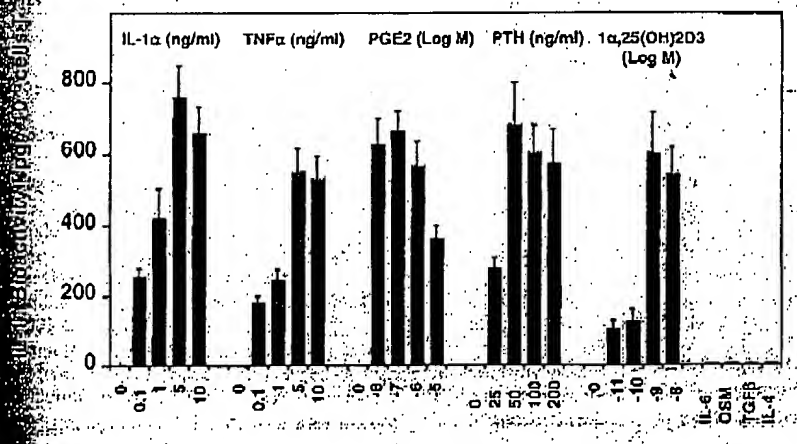

Figure 3. IL-11 production by primary osteoblasts. Subconfluent osteoblasts were cultured for 48 h in the absence (unstimulated) or presence of the indicated concentrations of mIL-1α, mTNFα, PGE_2, PTH, or $1\alpha,25(OH)_2D_3$. IL-11 released into the supernatants was quantitated using the Ba/F3 cell bioassay. Each value represents the IL-11 bioactivity (mean ± SE) of triplicate cultures. Neither rmIL-6 (20 ng/ml), rhOSM (20 ng/ml), rhTGFβ1 (1 ng/ml), or rmIL-4 (10 ng/ml) induced IL-11 release. The results are representatives of replicate experiments.

Table 1. *Specificity of the Ba/F3 Microassay for IL-11 in Osteoblast Culture Supernatants*

Culture supernatant (48 h)	αIL-11 mAb added (20 μg/ml) –	+
No stimulation	3 ± 1	0
IL-1 (5 ng/ml)	94 ± 9	6 ± 2*
TNFα (10 ng/ml)	90 ± 14	7 ± 3*
PGE_2 (10^{-6} M)	115 ± 16	9 ± 2*
hPTH (200 ng/ml)	82 ± 5	6 ± 2*
1α,25$(OH)_2D_3$ (10 nM)	63 ± 6	4 ± 2*

Transfected Ba/F3 cells were maintained for 48 h in the presence of culture supernatants (diluted 1:2) derived from primary osteoblastic cells treated for 48 h, without or with monoclonal murine anti-human IL-11 antibody (20 μg/ml). Values shown represent the mean ± SE number of viable Ba/F3 cells per well from four replicate wells.
* $P < 0.005$ vs cells cultured in the absence of monoclonal murine anti-human IL-11 antibody (αIL-11 mAb).

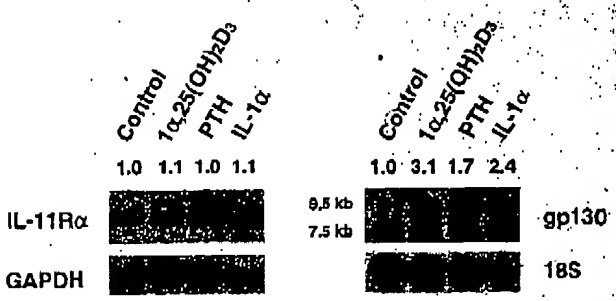

Figure 5. Expression of IL-11Rα and gp130 mRNAs by primary osteoblasts. Osteoblasts were cultured without additions (control) or with 1α,25 $(OH)_2D_3$ (10 nM), hPTH (200 ng/ml) or IL-1α (10 ng/ml) for 24 h. Total RNA was reverse transcribed and amplified by 30 cycles for IL-11Rα and 20 cycles for mouse GAPDH mRNA using the specific primers described in Methods. PCR products were transferred to a nylon membrane and hybridized with ^{32}P-labeled internal oligonucleotides specific for mIL-11Rα and GAPDH sequences respectively. For Northern analysis, total RNA (20 μg/lane) was hybridized to ^{32}P-labeled gp130 cDNA. Hybridization with ^{32}P-labeled oligonucleotide specific to 18S sequences was used to control for equivalence of loading. The number above each lane is the treated/control ratio of the intensity of the band(s) normalized to that of GAPDH or 18S RNA, measured by densitometry.

the anti-gp130 antibody abolished osteoclast formation induced by IL-1α (1 ng/ml) and inhibited, by between 60–75%, osteoclast formation induced by PGE_2 (10^{-6} M), 1α,25 $(OH)_2D_3$ (10 nM), or hPTH (1-34) (400 ng/ml) (Fig. 8) suggesting a common role of gp130 signals for osteoclastogenesis induced by several bone-resorbing factors. In these experiments, an equivalent concentration of non-immune IgG did not inhibit osteoclast formation (data not shown).

IL-11Rα Transcript Expression in Cells of the Osteoclast Lineage. To investigate the possibility that OCLs as well as bone marrow progenitors also express mRNA for IL-11Rα, the temporal relationship of the expression of IL-11Rα to CTR mRNA was studied during osteoclast formation in bone marrow cell cultures. As shown in Fig. 9, fresh bone marrow cells (day 0) constitutively expressed IL-11Rα transcripts. When cultures were stimulated by 10 nM 1α,25 $(OH)_2D_3$, a time-dependent increase in CTR mRNA (28 cycles of PCR amplification), relative to GAPDH mRNA (20 cycles of PCR amplification) occurred, reflecting differentiation of bone marrow cell progenitors, as mature osteoclasts were plentiful in the cultures after 5 d. More importantly, after 10 d, we consistently detected a parallel sevenfold rise in abundance of IL-11Rα transcripts (30 cycles of PCR amplification) relative to GAPDH mRNA, beginning at day 4 to 5. This phenomenon was relatively specific for IL-11Rα mRNA, as evidenced by the lack of appreciable change in abundance of IL-6Rα mRNA (25 cycles of PCR amplification) relative to GAPDH mRNA (Fig. 9). This sequential relationship between CTR mRNA and IL-11Rα mRNA expression suggested that expression of IL-11Rα mRNA was upregulated during OCL differentiation. To test this hypothesis, and to verify IL-11Rα gene expression by osteoblasts, we carried out in situ hybridization.

Cocultures grown on collagen gels were treated with 1α,25$(OH)_2D_3$ for 7 d, transferred to coverslips (18) and

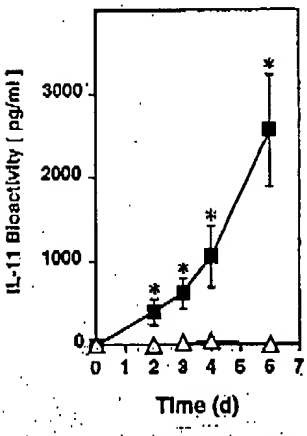

Figure 4. Effect of 1α,25-$(OH)_2D_3$ on IL-11 production in cocultures of primary osteoblasts and bone marrow cells. Cocultures were maintained for 6 d in the absence (unstimulated, -△-) or presence (—■—) of 1α,25 $(OH)_2D_3$ (10 nM), with a complete medium change after incubation for 3 d. At the times indicated, 50 μl of the culture medium was collected for quantitation of IL-11 using the Ba/F3 cell bioassay. The amount of IL-11 present 4 and 6 d after initiation of the culture was calculated by adding the IL-11 level obtained at day 3 to the absolute amounts determined at those times. Each point represents the mean cumulative IL-11 bioactivity of triplicate cultures. * $P < 0.005$ vs unstimulated cultures.

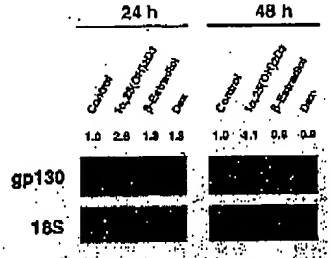

Figure 6. Effects of steroid hormones on gp130 mRNA expression by ST-2 cells. Subconfluent cells were cultured for 24 or 48 h under 2% serum conditions in phenol red free α-MEM without additions (control) or with 1α,25$(OH)_2D_3$ (10 nM), β-Estradiol (10 nM), or Dexamethasone (10^{-8} M, Dex). 20 μg/lane of fractionated total RNA was hybridized with ^{32}P-labeled gp130 cDNA, as described in Materials and Methods. The number in each lane is the treated/control ratio of the intensity of the bands normalized to that of 18S RNA, measured by densitometry.

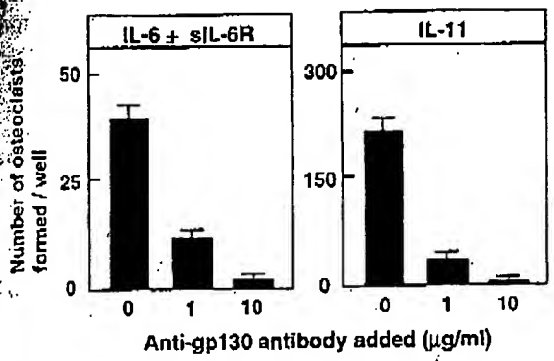

Figure 7. Effect of monoclonal anti-mouse gp130 antibody on OCL formation induced by mIL-6-sIL-6Rα, or mIL-11. Cocultures of bone marrow cells and primary osteoblasts were maintained in the presence of mIL-6 (20 ng/ml) in combination with smIL-6Rα (500 ng/ml), or mIL-11 (10 ng/ml). Simultaneously, graded concentrations of anti-gp130 antibody were added, as indicated. After culturing for 7 d, TRAP-positive cells were counted. Data are expressed as the mean ± SE total number of TRAP-positive cells (OCLs) per well of quadruplicate cultures. Pretreatment with non-immune IgG had no effect on osteoclast formation (data not shown). These results are representatives of four separate experiments.

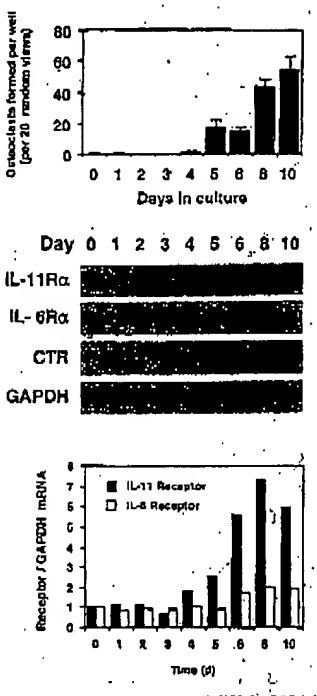

Figure 9. Effect of $1\alpha,25(OH)_2D_3$ on mIL-11Rα, mIL-6Rα, and mCTR mRNA expression in bone marrow cell cultures. Bone marrow cells were cultured for 10 d in the presence of $1\alpha,25(OH)_2D_3$ (10 nM). At each time point, osteoclasts were enumerated as described in Materials and Methods. RNA was reverse transcribed and subjected to 30 cycles of PCR for mIL-11Rα amplification, 25 cycles of PCR for mIL-6Rα mRNA amplification, 28 cycles of PCR for mCTR mRNA and 20 cycles of PCR for GAPDH mRNA, using specific primers. PCR products were resolved by electrophoresis, transferred to nylon membranes, and hybridized with specific [^{32}P]-labeled internal oligonucleotides as described in Materials and Methods. Upper panel shows timed appearance of osteoclasts in the cultures. Each value represents the mean ± SE of osteoclasts (cells exhibiting both TRAP staining and [^{125}I]-CT binding) per well from four replicate cultures. Middle panel shows PCR products for mIL-11Rα, mIL-6Rα, and CTR, at parallel time points. To allow semiquantitation of the PCR products, reverse transcribed RNA was also amplified using primers complementary to GAPDH sequences. The intensities of autoradiographic signals for mIL-11Rα, mIL-6Rα, and GAPDH PCR products were quantitated and are shown in the lower panel as the ratio of mIL-11Rα/GAPDH and mIL-6Rα/GAPDH. This figure is a representative of three separate experiments; similar results were obtained in the other two experiments.

then processed for in situ hybridization. As illustrated in Fig. 10 *A*, these cocultures contained IL-11Rα transcripts detectable by in situ hybridization. Indeed, both osteoblasts and OCLs expressed IL-11Rα mRNA. IL-11Rα transcripts were located predominantly in OCLs, which were identified by morphology and TRAP-staining (data not shown). The specificity of this result was verified by elimination of hybridization signals after pre-treatment with 100 μg/ml of ribonuclease for 1 h (Fig. 10 *B*).

Discussion

The present study focused on the regulation of IL-11 synthesis by primary osteoblasts and furthers our understanding of the role of gp130 signal transduction in osteoclast formation. IL-11 receptor transcripts were detected not only in osteoblasts, but in mature osteoclasts, indicating that both bone forming and bone resorbing cells are potential targets of IL-11.

Primary calvarial cell cultures consist of the heterogeneous osteoblastic-stromal cells that are likely to be present in the bone microenvironment in vivo. IL-11 production by primary osteoblasts was induced by IL-1, TNFα, PGE_2, PTH, and $1\alpha,25(OH)_2D_3$, all of which are potent osteoclastogenic factors in vitro. Unlike osteosarcoma cells (14), primary osteoblasts did not produce IL-11 constitutively. However, our data indicate that appropriately stimulated normal osteoblasts are a source of this cytokine. Although IL-11 was measured with a transfected cell line that is potentially sensitive to growth stimulation by IL-6-sIL-6Rα complexes (6), we did not detect sIL-6Rα in our experimental systems. We have previously shown that sIL-6Rα also is undetectable in murine cocultures despite dexamethasone stimulation, which greatly enhanced expression of osteoblast IL-6Rα mRNA and OCL formation (17).

Prostaglandin E2 was a potent inducer of IL-11 production by osteoblasts, and PGs seemed to mediate IL-11 prod-

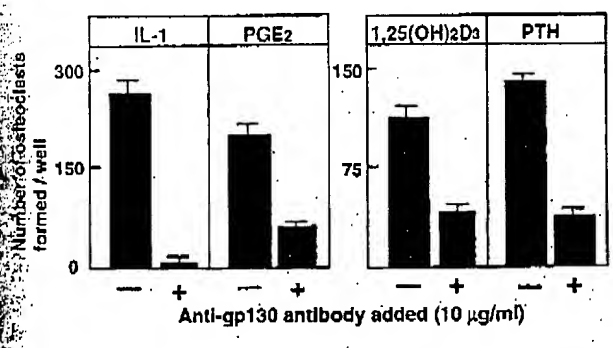

Figure 8. Effect of monoclonal anti-mouse gp130 antibody on osteoclast formation stimulated by IL-1, PGE_2, PTH, or $1\alpha,25(OH)_2D_3$. Cocultures of bone marrow cells and primary osteoblasts were maintained in the presence of IL-1α (1 ng/ml), $PGE_2(10^{-6}$ M), $1\alpha,25(OH)_2D_3$ (10 nM), or hPTH [1-34] (400 ng/ml), each without or with the indicated amounts of anti-gp130 antibody added simultaneously at the begining of the culture. After culturing for 7 d, TRAP-positive cells were counted. Data are expressed as the mean ± SE total number of TRAP-positive cells (OCLs) per well of quadruplicate cultures. Pretreatment with nonimmune IgG did not influence osteoclastogenesis (data not shown). These results are representatives of five separate experiments.

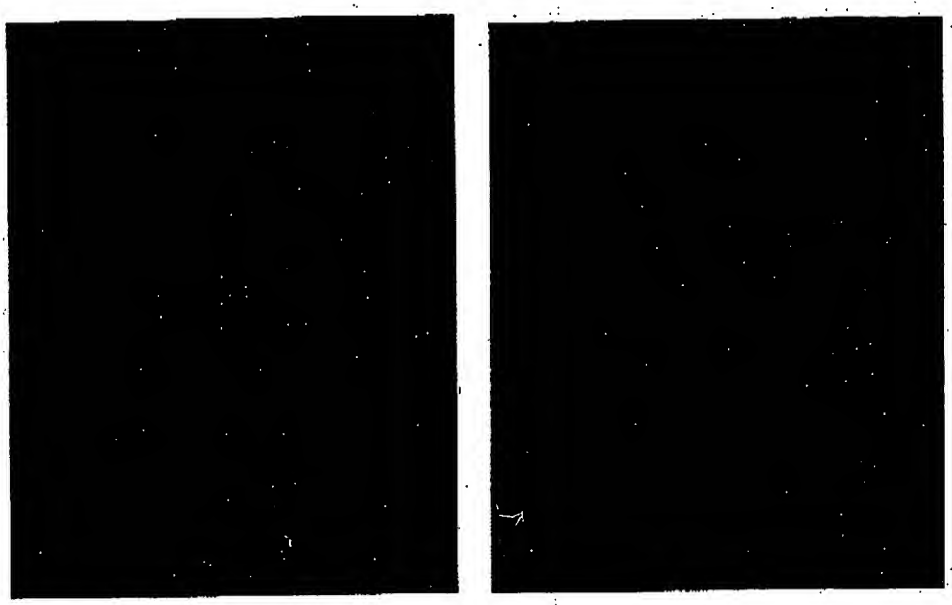

Figure 10. In situ hybridization. Cocultures of bone marrow cells and primary osteoblastic cells were subjected to in situ hybridization as detailed in Materials and Methods. (*A*) Photomicrograph of cells from the cocultures at day 7. The cells were hybridized with an anti-sense riboprobe specific for mIL-11Rα. Transcripts for IL-11Rα, represented by blue staining, are located in polygonal and spindle-shaped osteoblastic-stromal cells (*arrow heads*) and OCLs (*arrows*). (*B*) Specific hybridization signals were eliminated by pretreatment with RNase. All preparations were counterstained with nuclear fast red. Original magnification, ×240. The results shown in this figure were reproduced in three independent experiments.

duction stimulated by IL-1 and TNF in our primary osteoblast cultures. Prostaglandins are produced in bone by many cells, especially by osteoblasts, and production is stimulated by a variety of cytokines derived from macrophages or hemopoietic cells (34–38). The important role of PGs in IL-1-mediated osteoclast formation and bone resorption has been emphasized (39), and PGs appear to be essential for IL-11 mediated osteoclastogenesis as well (15). An IL-1 response element exists close to the IL-11 gene and, in certain stromal cells IL-1 can stimulate IL-11 by a mechanism independent of prostaglandins (40).

In the present study, the ability of $1\alpha,25(OH)_2D_3$ to potently stimulate IL-11 production was shown in osteoblasts and in cocultures of osteoblasts and bone marrow cells. These findings are consistent with a similar effect of $1\alpha,25(OH)_2D_3$ in bone marrow cell cultures (15). The concentrations of IL-11 present in supernatants of the co-cultures treated with $1\alpha,25(OH)_2D_3$ exceeded 1 ng/ml after 3 d, which was in accord with our previous estimate of the EC50 of exogenous IL-11 (~1.0 ng/ml) necessary for maximal osteoclastogenesis in such cocultures (16).

Cytokines exert their pleiotropic effects by interacting with specific cell surface receptors (11, 12). There is little direct information about the IL-11 receptor in connective tissue cells. During embryogenesis, primitive chondroblastic and osteoblastic progenitor cells seem to express higher levels of a transcript homologous with IL-11Rα compared to their more differentiated counterparts (13). Our results indicate that primary osteoblasts not only are a source of IL-11, but express transcripts for the complete IL-11 receptor complex (IL-11Rα and gp130). This evidence suggests that IL-11 acts in an autocrine-paracrine fashion. Stimulation of primary osteoblastic cells by $1\alpha,25(OH)_2D_3$, PTH or IL-1 upregulated gp130 mRNA up to threefold at 24 h, while mRNA levels for IL-11Rα were unchanged. Similar upregulation of gp130 mRNA (and protein) expression by $1\alpha,25(OH)_2D_3$ and PTH has been reported in murine MC3T3-E1 cells and primary bone marrow cell cultures (41). Indeed, it has been proposed that regulation of gp130 expression by systemic hormones could modulate the sensitivity of osteoblasts to cytokines such as IL-11 and IL-6-sIL-6Rα (41, 42). It is not known if gp130 may be rate-limiting for cytokine signal transduction, in the way that low levels of functional IL-6 receptors limit responsiveness of osteoblastic cells to IL-6 under physiological circumstances (16, 17). Expression of gp130 is ubiquitous (26), but it is possible that key target cells, such as bone marrow stromal cells might be relatively deficient in gp130 under certain conditions. Further studies are required to confirm this hypothesis.

We have reported that OCL formation is induced by at least three different mechanisms (32, 33). The first mechanism is the PTH-IL-1-PGE_2 axis, which is mediated by signaling involving cAMP. The second mechanism is $1\alpha,25(OH)_2D_3$-induced osteoclast formation, which is mediated by the vitamin D receptor but independent of cAMP. IL-1 induces OCL formation by a mechanism involving PG production (39). The gp130 signal, activated by cytokines such as IL-11, IL-6-sIL-6Rα, LIF, or OSM, is clearly an additional and important pathway of OCL formation. We previously showed that anti-IL-6Rα antibody inhibited OCL formation by IL-6-sIL-6Rα, but not by IL-1 or $1\alpha,25(OH)_2D_3$ (16). This indicates that IL-6 is not implicated in osteoclastogenesis stimulated by IL-1 or $1\alpha,25(OH)_2D_3$. In the present study, neutralizing anti-gp130 antibody, abolished osteoclast formation induced by IL-1. Moreover, the antibody partially inhibited the osteoclastogenic effects of PGE_2, PTH, and $1\alpha,25(OH)_2D_3$. This indicates that gp130 signals, probably evoked by IL-11, are indispensable for IL-1-induced OCL formation. This is consistent with the ability

of IL-1 to induce both PGE_2 and IL-11 production by osteoblasts. Similarly, we speculate that IL-11 contributes, at least in part, to OCL formation induced by PGE_2, PTH, and $1\alpha,25(OH)_2D_3$. Girasole et al. also reported that a monoclonal anti-IL-11 antibody inhibited PTH-, $1\alpha,25(OH)_2D_3$-, IL-1-, or TNFα-mediated OCL formation by between 50–100% (15).

Together, these results suggest that the gp130 signal is a pivotal cofactor for osteoclast formation. Targeted disruption of gp130 may clarify the physiological role of IL-11 and related cytokines in osteoclast formation and skeletal development (43).

Osteotropic factors appear to act directly on osteoblastic cells, which in turn produce a factor responsible for osteoclast differentiation (17, 32). This putative factor may be expressed on the cell surface membranes and plays a critical role through a juxtacrine (cell-to-cell contact) mechanism. In support of this concept, we recently established that the ability of IL-6 to induce osteoclast differentiation depends on signal transduction mediated by IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors (17). In view of this evidence, it is likely that IL-11 also induces osteoclast formation by activating gp130 signals via IL-11 receptors present on osteoblasts. In bone marrow cell cultures, which are relatively deficient in stromal cells, exogenous IL-11 is insufficient to trigger osteoclastogenesis (15), but augments the effect of $1\alpha,25(OH)_2D_3$, perhaps because gp130 is upregulated under these conditions.

The demonstration of IL-11Rα mRNA expression in mature OCLs suggests an important biological function of IL-11 in osteoclasts, perhaps distinct from its role in osteoclast formation. Ohsaki et al. (44) demonstrated functional IL-6 receptors in human osteoclast-like cells derived from giant cell tumors of bone. In these cells, IL-6 appeared to modulate bone resorbing activity. However, anti-gp130 antibody did not influence pit resorption by OCLs (data not shown). Macrophage colony stimulating factor and IL-1 appear to promote survival of OCLs (45), but we found that IL-11 itself did not support OCL survival (data not shown). Further studies will be required to demonstrate functional IL-11 receptors on OCLs and elucidate the role of IL-11 in mature osteoclasts.

In conclusion, normal osteoblasts are a source of IL-11, the production of which is induced by IL-1, TNF, PGE_2, PTH, and $1\alpha,25(OH)_2D_3$. Bone resorbing hormones and IL-1 also upregulate gp130 mRNA in these cells and consequently cell responsiveness to IL-11 and related cytokines may be enhanced. Both osteoblasts and mature OCLs express IL-11Rα mRNA and thus are potentially able to respond to IL-11. Signals mediated by gp130 may be involved in physiological regulation of osteoclast formation stimulated by osteotropic factors. Since high local concentrations of IL-1, TNF, and PGs occur in rheumatoid arthritis synovium (46), IL-11 could contribute to the excessive osteoclastic bone resorption observed in that disease.

We thank Dr. M. Ikegame for preparation of bone marrow cell cultures and Dr. N. Nicola (Walter and Eliza Hall Institute, Melbourne) for helpful discussions.

This work was supported by a Program Grant from the National Health and Medical Research Council (NH and MRC) of Australia, a Grant-in-aid from the Arthritis Foundation of Australia and Grants-in-aid 06404067 and 06771640 from the Ministry of Science, Education and Culture of Japan. E. Romas is supported by a Medical Postgraduate NH and MRC Scholarship. N. Udagawa is a C.R. Roper Research Fellow, University of Melbourne.

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Received for publication 18 December 1995 and in revised form 3 April 1996.

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